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Role of JNK1-dependent Bcl-2 Phosphorylation in Ceramide-induced Macroautophagy*[S]**

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Macroautophagy is a vacuolar lysosomal catabolic pathway that is stimulated during periods of nutrient starvation to preserve cell integrity. Ceramide is a bioactive sphingolipid associated with a large range of cell processes. Here we show that short-chain ceramides (C_2-ceramide and C_6-ceramide) and stimulation of the *de novo* ceramide synthesis by tamoxifen induce the dissociation of the complex formed between the autophagy protein Beclin 1 and the anti-apoptotic protein Bcl-2. This dissociation is required for macroautophagy to be induced either in response to ceramide or to starvation. Three potential phosphorylation sites, Thr^{69}, Ser^{70}, and Ser^{87}, located in the non-structural N-terminal loop of Bcl-2, play major roles in the dissociation of Bcl-2 from Beclin 1. We further show that activation of c-Jun N-terminal protein kinase 1 by ceramide is required both to phosphorylate Bcl-2 and to stimulate macroautophagy. These findings reveal a new aspect of sphingolipid signaling in up-regulating a major cell process involved in cell adaptation to stress.

Macroautophagy (referred to below as "autophagy") is a vacuolar, lysosomal degradation pathway for cytoplasmic constituents that is conserved in eukaryotic cells (1–3). Autophagy is initiated by the formation of a multimembrane-bound autophagosome that engulfs cytoplasmic proteins and organelles. The last stage in the process results in fusion with the lysosomal compartments, where the autophagic cargo undergoes degradation. Basal autophagy is important in controlling the quality of the cytoplasm by removing damaged organelles and protein aggregates. Inhibition of basal autophagy in the brain is deleterious, and leads to neurodegeneration in mouse models (4, 5). Stimulation of autophagy during periods of nutrient starvation is a physiological response present at birth and has been shown to provide energy in various tissues of newborn pups (6). In cultured cells, starvation-induced autophagy is an autonomous cell survival mechanism, which provides nutrients to maintain a metabolic rate and level of ATP compatible with cell survival (7). In addition, starvation-induced autophagy blocks the induction of apoptosis (8). In other contexts, such as drug treatment and a hypoxic environment, autophagy has also been shown to be cytoprotective in cancer cells (9, 10). However, autophagy is also part of cell death pathways in certain situations (11). Autophagy can be a player in apoptosis-independent type-2 cell death (type-1 cell death is apoptosis), also known as autophagic cell death. This situation has been shown to occur when the apoptotic machinery is crippled in mammalian cells (12, 13). Autophagy can also be part of the apoptotic program, for instance in tumor necrosis factor-α-induced cell death when NF-κB is inhibited (14), or in human immunodeficiency virus envelope-mediated cell death in bystander naive CD4 T cells (15). Moreover autophagy has recently been shown to be required for the externalization of phosphatidylserine, the eat-me signal for phagocytic cells, at the surface of apoptotic cells (16).

The complex relationship between autophagy and apoptosis reflects the intertwined regulation of these processes (17, 18). Many signaling pathways involved in the regulation of autophagy also regulate apoptosis. This intertwining has recently been shown to occur at the level of the molecular machinery of autophagy. In fact the anti-apoptotic protein Bcl-2 has been shown to inhibit starvation-induced autophagy by interacting with the autophagy protein Beclin 1 (19). Beclin 1 is one of the Atg proteins conserved from yeast to humans (it is the mammalian orthologue of yeast Atg6) and is involved in autophagosome formation (20). Beclin 1 is a platform protein that interacts with several different partners, including hVps34 (class III phosphatidylinositol 3-kinase), which is responsible for the synthesis of phosphatidylinositol 3-phosphate. The production of this lipid is important for events associated with the nucleation of the isolation membrane before it elongates and closes to form autophagosomes in response to other Atg proteins, including the Atg12 and LC3[2] (microtubule-associated protein light chain 3

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[2] The abbreviations used are: LC3, Light Chain 3; C_2-Cer, C_2-ceramide; C_2-DHCer, C_2-dihydroceramide; C_6-Cer, C_6-ceramide; C_6-DHCer, C_6-dihydroceramide; DAG, diacylglycerol; dnJNK1, dominant-negative JNK1; FB1, fumonisin B1; EBSS, Earle's balanced salt solution; Tam, tamoxifen; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; CM, complete medium; mTOR, mammalian target of rapamycin.

is the mammalian orthologue of the yeast Atg8) ubiquitin-like conjugation systems (3, 21). Various partners associated with the Beclin 1 complex modulate the activity of hVps34. For instance, Bcl-2 inhibits the activity of this enzyme, whereas UVRAG, Ambra-1, and Bif-1 all up-regulate it (22, 23).

In view of the intertwining between autophagy and apoptosis, it is noteworthy that Beclin 1 belongs to the BH3-only family of proteins (24–26). However, and unlike most of the proteins in this family, Beclin 1 is not able to trigger apoptosis when its expression is forced in cells (27). A BH3-mimetic drug, ABT-737, is able to dissociate the Beclin 1-Bcl-2 complex, and to trigger autophagy by mirroring the effect of starvation (25).

The sphingolipids constitute a family of bioactive lipids (28–32) of which several members, such as ceramide and sphingosine 1-phosphate, are signaling molecules. These molecules constitute a "sphingolipid rheostat" that determines the fate of the cell, because in many settings ceramide is pro-apoptotic and sphingosine 1-phosphate mitigates this apoptotic effect (31, 32). However, ceramide is also engaged in a wide variety of other cell processes, such as the formation of exosomes (33), differentiation, cell proliferation, and senescence (34). Recently we showed that both ceramide and sphingosine 1-phosphate are able to stimulate autophagy (35, 36). It has also been shown that ceramide triggers autophagy in a large panel of mammalian cells (37–39). However, elucidation of the mechanism by which ceramide stimulates autophagy is still in its infancy. We have previously demonstrated that ceramide induces autophagy in breast and colon cancer cells by inhibiting the Class I phosphatidylinositol 3-phosphate/mTOR signaling pathway, which plays a central role in inhibiting autophagy (36). Inhibition of mTOR is another hallmark of starvation-induced autophagy (17). This finding led us to investigate the effect of ceramide on the Beclin 1-Bcl-2 complex. The results presented here show that ceramide is more potent than starvation in dissociating the Beclin 1-Bcl-2 complex (see Ref. 40). This dissociation is dependent on three phosphorylation sites (Thr^{69}, Ser^{70}, and Ser^{87}) located in a non-structural loop of Bcl-2. Ceramide induces the c-Jun N-terminal kinase 1-dependent phosphorylation of Bcl-2. Expression of a dominant negative form of JNK1 blocks Bcl-2 phosphorylation, and thus the induction of autophagy by ceramide. These findings help to explain how autophagy is regulated by a major lipid second messenger.

MATERIALS AND METHODS

Reagents—C_2-Cer, C_6-Cer, C_2-DHCer, and C_6-DHCer were from Sigma and were dissolved in ethanol before use. FB1, Myriocin, and TAM were purchased from Biomol. Cell culture medium, Lipofectamine 2000, and fetal bovine serum were from Invitrogen. The radioisotope L-[U-^{14}C]valine (256 mCi/mmol), the ECL™ Western blotting detection kit, and the donkey anti-rabbit antibody were purchased from Amersham Biosciences. Goat anti-mouse and swine anti-goat antibodies were obtained from Bio-Rad and Caltag (Burlingame, CA), respectively. Mouse monoclonal anti-p62 antibody was obtained from BD Biosciences. Mouse monoclonal anti-Bcl-2 and goat polyclonal anti-Beclin 1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit monoclonal antibodies against p-Bcl-2, p-JNK, and total JNK were obtained from Cell Signaling. Rabbit polyclonal anti-Beclin 1 antibody was obtained from Novus Biologicals. Rabbit polyclonal anti-LC3 antibody was obtained as previously described (41).

Cell Culture—Human breast cancer cell line MCF-7 cells stably transfected with *beclin 1* (MCF7.*beclin 1*) were cultured as previously described (42). HeLa cells were obtained from ATCC, and maintained at 37 °C in 10% CO_2 in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 100 ng/ml each of penicillin and streptomycin. HeLa GFP-LC3 cells, kindly provided by A. M. Tolkovsky (University of Cambridge, UK), were cultured in the presence of 200 μg/ml G418. The human colon cancer cell line HT-29 transfected either with empty vector or vector encoding Bcl-2 were kindly provided by M. T. Dimanche-Boitrel (INSERM U620, France), and cultured at 37 °C in 10% CO_2 in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and 100 ng/ml each of penicillin and streptomycin plus 200 μg/ml G418. MEF WT and Atg5−/− were kindly given by N. Mizushima (Tokyo Medical and Dental University, Japan), and cultured as previously described (6). The trypan blue exclusion test showed that cell viability was greater than 90% under all the experimental conditions used.

Quantification of Endogenous Ceramide—Ceramide was determined using *Escherichia coli* DAG kinase as previously reported (43). The *E. coli* strain was kindly provided by Drs. D. K. Perry and Y. A. Hannun (Medical University of South Carolina, Charleston, SC).

Beclin 1 and Bcl-2 Co-immunoprecipitation—To immunoprecipitate endogenous Beclin 1 and endogenous Bcl-2 in HeLa cells or stably transfected Beclin 1 in MCF.7 cells, the cells were lysed in CHAPS lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 10% glycerol, and 2% CHAPS) for 3 h at 4 °C, and immunoprecipitation was performed overnight at 4 °C with a goat polyclonal antibody (1:80 dilution, Santa Cruz Biotechnology). Protein A-Sepharose beads (Amersham Biosciences) were added for 2 h at 4 °C, washed twice with 137 mM NaCl CHAPS wash buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 10% glycerol, and 0.5% CHAPS), and twice with 274 mM NaCl CHAPS wash buffer. Anti-Beclin 1 immunoprecipitates were subjected to SDS-PAGE, and Bcl-2 was detected by immunoblot analysis (19).

GFP-LC3 Assay—The assay was performed in HeLa cells either stably transfected with rat GFP-LC3 (kindly provided by T. Yoshimori, Osaka University, Japan) or in transiently transfected with MCF-7. *beclin 1*, HT-29, and HeLa cells using Lipofectamine 2000 (19, 42). HeLa cells were also transfected either with human GFP-LC3B or the mutant GFP-LC3BΔG (kindly provided by I. Tanida, National Institute of Infectious Disease, Tokyo, Japan). The vector, pcDNA3-FLAG-MKK7-JNK1(APF) (dominant-negative JNK1, dnJNK1), was provided by R. J. Davis (Howard Hughes Medical Institute) (44). When required, co-transfection with a plasmid encoding GFP-LC3 and a vector encoding for dnJNK1, or the empty vector pcDNA3, was performed in MCF-7.*beclin 1* cells (ratio GFP-LC3:vector, 1:3). Prior to analysis, the cells were starved for 4 h in Earle's balanced salt solution (EBSS, starvation medium), maintained in

Dulbecco's modified Eagle's medium with 10% fetal calf serum (control medium), or treated as described in the text. Autophagy was then measured by light microscopic counting of cells with GFP-LC3 puncta as described previously (42). A minimum of 50–100 cells per sample was counted in triplicate samples per condition per experiment.

Analysis of Protein Degradation—HT-29 cells were incubated for 24 h at 37 °C with 0.2 μCi/ml L-[^{14}C]valine. Three hours before the end of the radiolabeling period, cells were exposed to increasing concentrations of C_2-Cer or C_2-DHCer, and when required 100 nM FB1 was added, also 3 h before the end of radiolabeling. At the end of radiolabeling period, the cells were washed three times with PBS, pH 7.4. Cells were then incubated in complete medium supplemented with 10 mM cold valine. After incubating for 1 h, by which time short-lived proteins are degraded, the medium was replaced with fresh nutrient-free medium (EBSS plus 0.1% of bovine serum albumin and 10 mM cold valine), and the incubation was continued for an additional 4 h. Cells and radiolabeled proteins from the 4-h chase medium were precipitated in trichloroacetic acid at a final concentration of 10% (v/v) at 4 °C. The precipitated proteins were separated from the soluble radioactivity by centrifugation at 600 × *g* for 10 min, and then dissolved in 0.5 ml of 0.2 N NaOH. Radioactivity was determined by liquid scintillation counting. Protein degradation was calculated by dividing the acid-soluble radioactivity recovered from both cells and medium by the radioactivity contained in precipitated proteins from both cells and medium (45).

Immunoblotting—After being resolved by SDS-PAGE, proteins were transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non fat dry milk in PBST (PBS and 0.1% Tween 20) for 1 h at room temperature, and then incubated with the appropriate primary antibody overnight at 4 °C in PBST. The antibody dilutions were as follows: anti-LC3 1:10,000; anti-JNK1-P(Thr^{183}/Tyr^{185}), anti-JNK1, anti-Bcl-2-P(Ser^{70}), anti-Bcl-2 1:1000; anti-p62 1:2000; anti-Beclin 1 1:2000; anti-actin 1:5000). After three washes in PBST, the membrane was incubated for 1 h at room temperature with the appropriate horseradish peroxidase-labeled secondary antibody. Bound antibodies were detected using ECL.

Statistical Analysis—Statistical analysis of the differences between the groups was performed using Student's *t* test. $p < 0.05$ was considered statistically significant.

RESULTS

Ceramide Induces Autophagy in Several Cell Lines—We have previously reported that the permeant short-chain C_2-ceramide (C_2-Cer) induces autophagy both in MCF-7 breast cancer cells and in HT-29 colon carcinoma cells via the production of endogenous long-chain ceramides (36). Autophagy was monitored by electron microscopy and by measuring the autophagic flux by analyzing the rate of long-lived protein degradation-sensitive to 3-methyladenine, an inhibitor of the formation of autophagosomes (46). In a first series of experiments, we extended these findings to HeLa GFP-LC3 cells by analyzing the formation of GFP-LC3 puncta. During autophagy, the LC3 protein is relocated to the autophagosomal membranes as a result of C-terminal conjugation to phosphatidylethanolamine.

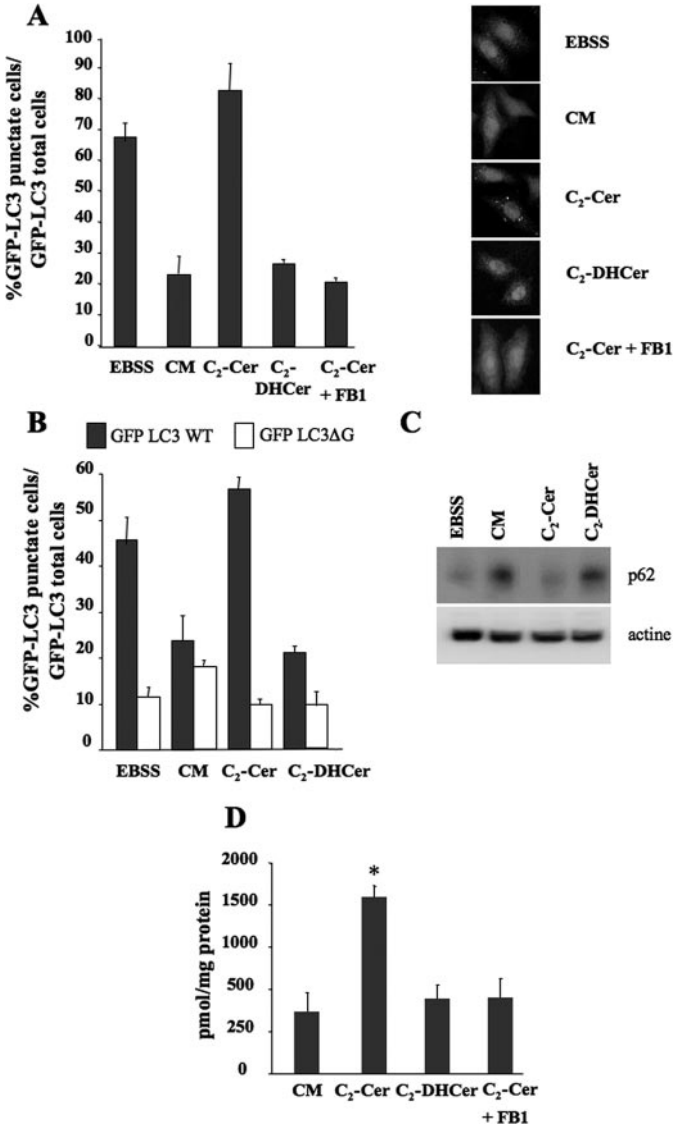

FIGURE 1. **C_2-Cer induces autophagy in HeLa cells.** *A*, HeLa GFP-LC3 cells were cultured for 4 h in either EBSS or complete medium (*CM*) either alone or supplemented with 100 μM C_2-Cer or 100 μM C_2-DHCer. *Left panel*, autophagy was quantified by counting the number of cells with GFP-LC3 puncta. The result shown is representative of three independent experiments. 50–100 cells were analyzed per assay. *Right panel*, representative images. *B*, HeLa cells were transfected with a plasmid encoding human GFP-LC3 WT or human GFP-LC3ΔG. 24 h after transfection, the cells were placed for 4 h in either EBSS or complete medium (*CM*), either alone or supplemented with 100 μM C_2-Cer or 100 μM C_2-DHCer. Autophagy was quantified by counting the number of cells with GFP-LC3 or GFP-ΔG puncta. The result shown is representative of three independent experiments. 50–100 cells were analyzed per assay. The result shown is representative of three independent experiments. *C*, HeLa cells were placed for 4 h in EBSS, CM, 100 μM C_2-Cer, or 100 μM C_2-DHCer. 10 μg of lysates was subjected to immunoblotting using anti-p62 antibody (1:2000) or anti-actin antibody (1:5000). *D*, determination of endogenous long-chain ceramides by the DAG method described under "Materials and Methods." Values reported are the mean ± S.D. of three independent experiments. *, $p < 0.05$ *versus* CM.

Thus, the accumulation of GFP-LC3 puncta provides an effective way of detecting autophagosomes (47). C_2-Cer treatment induced the accumulation of GFP-LC3 puncta in HeLa GFP-LC3 cells, whereas treatment with C_2-dihydroceramide (C_2-DHCer), a C_2-Cer analogue that does not induce autophagy in MCF-7 and HT-29 cells (36), failed to do so (Fig. 1*A*). To check that the formation of GFP-LC3 puncta in response to C_2-Cer

was not simply due to clumping of the chimeric protein induced by C_2-Cer treatment, we repeated the experiment in cells transfected with the mutant GFP-LC3ΔG, which is unable to support the formation of autophagosomes (48). Under these conditions, the mutant chimeric protein did not form puncta in response to C_2-Cer treatment (Fig. 1*B*). Furthermore, C_2-Cer was also unable to induce GFP-LC3 puncta in Atg5$^{-/-}$ MEF cells (supplemental Fig. S1*A*) that lack the essential autophagy protein Atg5 (49). Unlike C_2-Cer, C_2-DHCer did not induce autophagy in wt MEF (supplemental Fig. S1*A*). The accumulation of GFP-LC3 puncta was also observed in MCF-7.*beclin 1* cells. This cell line was engineered from a MCF-7 cell population with a low level of Beclin 1 expression and provides a convenient tool to for investigating autophagy, because the expression of Beclin 1 is under the control of a Tet-OFF system (50). In the absence of tetracycline we observed that C_2-Cer, but not C_2-DHCer, induced the formation of GFP-LC3 puncta (supplemental Fig. S2*A*). To confirm that the formation of GFP-LC3 puncta induced by C_2-Cer was indeed attributable to stimulation of the autophagic pathway, *i.e.* to increases in both the formation of autophagosomes and their consumption by the lysosomal compartment, we analyzed the effect of C_2-Cer on two independent assays of autophagic flux, the degradation of p62 and that of [^{14}C]valine-labeled long-lived protein. In HeLa GFP-LC3 cells, we observed that protein p62, a substrate for starvation-induced autophagy (51), was just as well degraded in nutrient-free medium (EBSS) as in C_2-Cer-treated cells (Fig. 1*C*). In contrast, cells treated with either complete media (CM) or C_2-DHCer were unable to stimulate p62 degradation (Fig. 1*C*). In MCF-7.*beclin 1* cells, C_2-Cer treatment increased the degradation of long-lived proteins sensitive to 3-methyladenine (supplemental Fig. S2*B*).

Our previous results had shown that the induction of autophagy depends on the elongation of C_2-Cer to form long chain ceramides (36). In HeLa GFP-LC3 (Fig. 1*D*), MEF (supplemental Fig. S1*B*) and MCF-7.*beclin 1* cells (supplemental Fig. S2*C*) an accumulation of long-chain ceramide was observed after C_2-Cer treatment. The elongation step depends on the activity of ceramide synthase is sensitive to FB1 (52). In all the cell lines used, FB1 treatment (100 nM) blocked both the elongation of C_2-Cer and C_2-Cer-induced autophagy (Fig. 1 and supplemental Figs. S1 and S2). In line with our previous findings (35, 36), these results strongly suggest that endogenous long-chain ceramides are potent stimulators of autophagy.

To further substantiate the role of long-chain ceramides in autophagy, we repeated the above experiments using the short chain C_6-Cer, which is a good substrate for generating the sphingosine backbone for long-chain ceramides (53). We found that exposing HeLa GFP-LC3 cells to 100 μM of C_6-Cer (for 4 h) significantly increased autophagy assessed by counting the number of puncta per cell (Fig. 2*A*). In contrast, its inactive counterpart, C_6-DHCer was unable to regulate autophagy (Fig. 2*A*). During the course of these experiments we observed an accumulation of long-chain ceramides in C_6-Cer-treated cells. Once again FB1 treatment blocked both the elongation of C_6-Cer (Fig. 2*C*) and C_6-Cer-induced autophagy (data not shown). Stimulation of autophagy and accumulation of long-chain ceramides were also observed when C_6-Cer was used at a

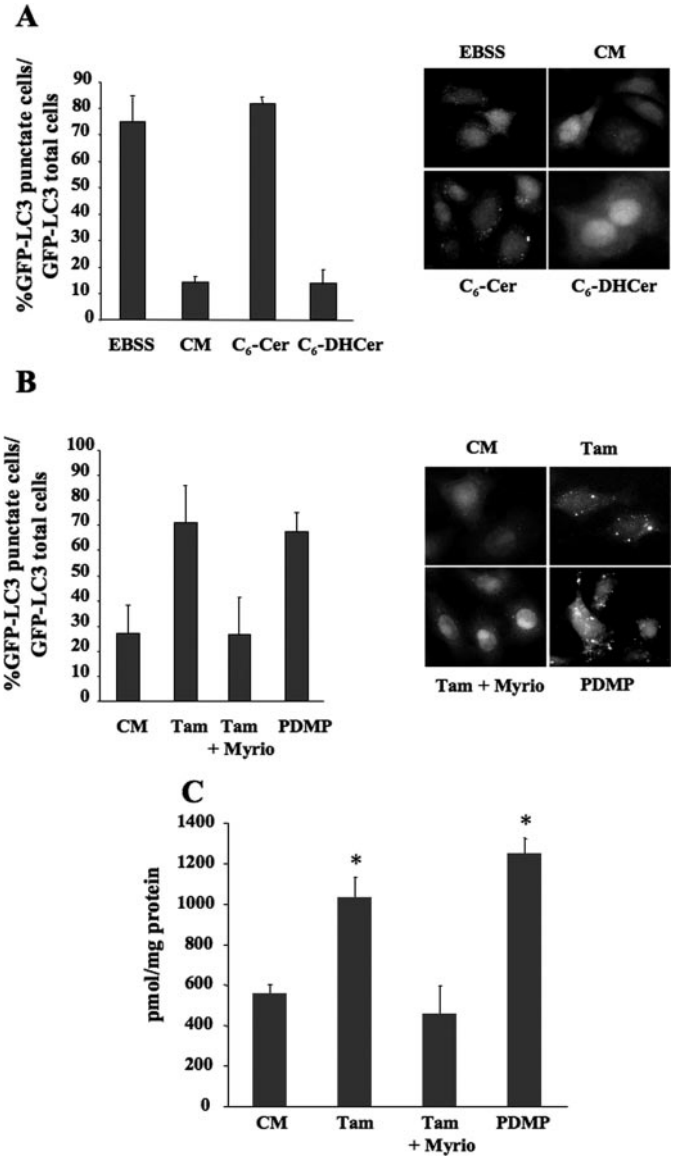

FIGURE 2. **C_6-Cer induces autophagy in HeLa cells.** *A*, HeLa GFP-LC3 cells were cultured for 4 h in either EBSS or complete medium (*CM*), either alone or supplemented with 100 μM C_6-Cer or C_6-DHCer. *Left panel*, autophagy was quantified by counting the number of cells with GFP-LC3 puncta. The result shown is representative of three independent experiments. 50–100 cells were analyzed per assay. *Right panel*, representative images. *B*, HeLa GFP-LC3 cells were cultured for 24 h with CM, 1 μM Tam, or 1 μM Tam plus 100 nM Myriocin, or 2.5 μM 1-phenyl-2-decanoylamino-3-morpholino-1-propanol. *Left panel*, autophagy was quantified by counting the number of cells with GFP-LC3 puncta. The result shown is representative of three independent experiments. 50–100 cells were analyzed per assay. The result shown is representative of three independent experiments. *Right panel*, representative images. *C*, quantitation of endogenous long-chain ceramides, by the DAG as described under "Materials and Methods." Values reported are the mean ± S.D. of three independent experiments. *Asterisks* indicate $p < 0.05$ *versus* CM.

lower concentration (40 μM) for different periods of time (supplemental Fig. S3). C_6-Cer also increased the autophagic flux as determined by analyzing the rate of long-lived protein degradation (data not shown). We have previously shown that Tamoxifen (Tam) and 1-phenyl-2-decanoylamino-3-morpholino-1-propanol stimulate autophagy in MCF-7 by increasing the level of endogenous long-chain ceramides (36). These treatments both increased the formation of long-chain ceramides presumably either by stimulating the *de novo* ceramide synthesis in the

ER, or by inhibiting the conversion of ceramide to glucosylceramide (36, 54). Here we show that Tam and 1-phenyl-2-decanoylamino-3-morpholino-1-propanol stimulated autophagy (Fig. 2*B*) and increased the formation of long-chain ceramides (Fig. 2*C*) in HeLa-GFP-LC3 cells. Moreover, Myriocin, a potent inhibitor of serine palmitoyltransferase the key rate-limiting enzyme of the *de novo* synthesis of ceramide (52), blocked both autophagy and the accumulation of long-chain ceramides in Tam-treated HeLa GFP-LC3 cells (Fig. 2, *B* and *C*).

In a previous study, we demonstrated that ceramide stimulates autophagy by interfering with the activation of Akt/PKB upstream of mTOR, a key regulator of autophagy signaling (36). However, we wonder whether ceramide could also stimulate autophagy by directly modulating the activity of the Atg machinery involved in autophagosome formation.

Ceramide Induces Dissociation of the Beclin 1-Bcl-2 Complex—Autophagy is tightly regulated by the activity of the Beclin 1 complex in initiating the formation of autophagosomes (22, 23). In this complex, the anti-apoptotic protein Bcl-2 represses autophagy. Dissociation of the Beclin 1-Bcl-2 complex stimulates autophagy whether induced by starvation or in response to BH3 mimetic molecules (19, 25). However, the role of lipid mediators in the regulation of the Beclin 1-Bcl-2 complex is unknown. We used HeLa cells that express detectable endogenous levels of both Beclin 1 and Bcl-2 to investigate the effect of ceramide on the dissociation of Beclin 1 and Bcl-2. Similarly to starvation (see Ref. 19 and Fig. 3*A*), co-immunoprecipitation experiments showed that, when C_2-Cer or C_6-Cer were added to cells, dissociation of the Beclin 1-Bcl-2 complex was observed together with the induction of autophagy. C_2-DHCer and C_6-DHCer did not stimulate autophagy under the experimental conditions used here, nor did they dissociate the complex (Fig. 3*A*).

To confirm that long-chain ceramides are indeed responsible for the dissociation of the Beclin 1-Bcl-2 complex, we next investigated the effects of Tam. We observed a dissociation of the Beclin 1-Bcl-2 complex in Tam-treated cells that correlated with the accumulation of GFP-LC3 puncta (Fig. 3*B*). Moreover, when Myriocin, an inhibitor of serine palmitoyl transferase, a key enzyme in ceramide biosynthesis (52), was present, Tam no longer induced dissociation of the Beclin 1-Bcl-2 complex; *i.e.* Myriocin reduced the formation of GFP-LC3 puncta by Tam-treated cells (Fig. 3*B*). From these findings, we conclude that the accumulation of long-chain ceramide stimulates autophagy by promoting dissociation of the Beclin 1-Bcl-2 complex.

Effect of Bcl-2 Expression on the Beclin 1-Bcl-2 Complex—We next wanted to find out whether modulation of Bcl-2 expression mitigates the autophagic response to C_2-Cer. To do this, we used the HT-29 colon carcinoma cell line, which we had previously reported to be sensitive to starvation-induced autophagy and to C_2-Cer-induced autophagy (36, 55). Moreover, this cell line expresses Beclin 1, but no detectable level of Bcl-2 (19). In accordance with previously published results (19), when Bcl-2 was stably expressed in this cell line, a blockade of the Beclin 1-Bcl-2 complex was observed (Fig. 4*A*) as well as inhibition of starvation-induced autophagy, as revealed by the presence of fewer GFP-LC3 puncta (Fig. 4*C*), and no stimulation of long-lived protein degradation (Fig. 4*B*). In contrast, C_2-Cer was able to trigger autophagy, as shown by an increase

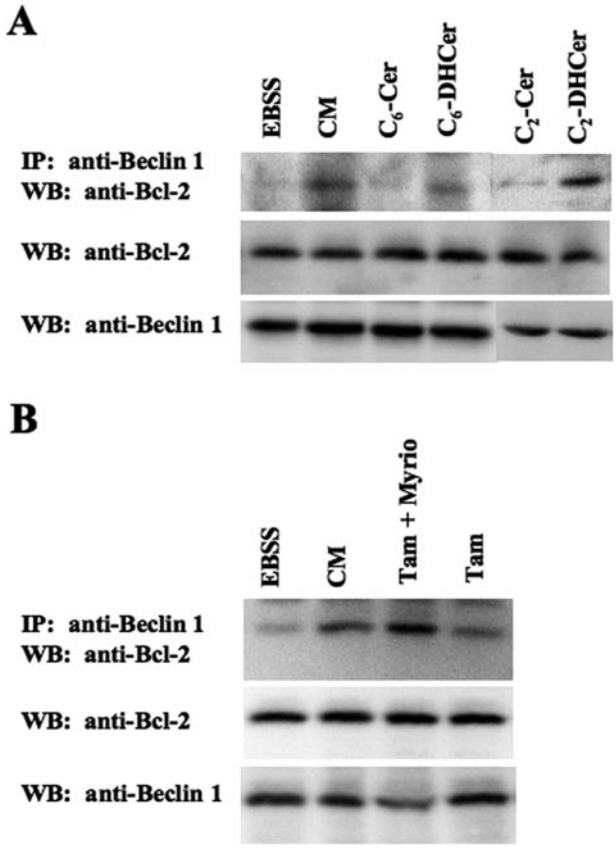

FIGURE 3. **Ceramide induces Beclin 1-Bcl-2 complex dissociation in HeLa cells.** *A*, HeLa cells were cultured for 4 h in either EBSS or complete medium (*CM*), either alone or supplemented with 100 μM C_6-Cer, C_6-DHCer, C_2-Cer, or C_2-DHCer. Endogenous Beclin 1 was immunoprecipitated using goat polyclonal antibody (1:80 dilution). Immunoprecipitated proteins were subjected to immunoblotting using a polyclonal anti-Bcl-2 antibody. Lysates were immunoblotted using a polyclonal anti-Bcl-2 or a polyclonal anti-Beclin 1 antibodies. *B*, HeLa cells were cultured for 24 h in either EBSS or CM, either alone or supplemented with 1 μM Tam, or 1 μM Tam plus 100 nM Myriocin. Endogenous Beclin 1 was immunoprecipitated using goat polyclonal antibody (1:80 dilution). Immunoprecipitated proteins were subjected to immunoblotting using a polyclonal anti-Bcl-2 antibody. Lysates was immunoblotted using a polyclonal anti-Bcl-2 or a polyclonal anti-Beclin 1 antibody. Western blots are representative of three independent experiments.

in the number of GFP-LC3 puncta, and stimulated long-lived protein degradation in HT-29-Bcl-2 cells (Fig. 4, *B* and *C*). Stimulation of autophagy by C_2-Cer in HT-29-Bcl-2 cells was characterized by the dissociation of the Beclin 1-Bcl-2 complex (Fig. 4*A*). In contrast to C_2-Cer, C_2-DHCer neither induced the dissociation of the Beclin 1-Bcl-2 complex (Fig. 4*A*) nor triggered autophagy in HT-29-Bcl-2 cells (Fig. 4, *B* and *C*). These results suggest that, under conditions where Bcl-2 blunts starvation-induced autophagy, ceramide is able to overcome this inhibitory effect. These results were confirmed by analyzing the dissociation of the Beclin 1-Bcl-2 complex, and the formation of GFP-LC3 puncta in MCF-7.*beclin 1* cells after transfection with a Myc-tagged form of Bcl-2 (Fig. 5). In this setting, starvation did not lead to dissociation of the Beclin 1-Bcl-2 complex but did stimulate the formation of GFP-LC3 puncta when compared with untransfected cells (see Refs. 19, 40 and Fig. 5 (*B* and *C*)). In contrast, C_2-Cer did trigger both autophagy and Beclin 1-Bcl-2 complex dissociation in MCF-7.*beclin 1* cells expressing Myc-Bcl-2 (Fig. 5, *B* and *C*).

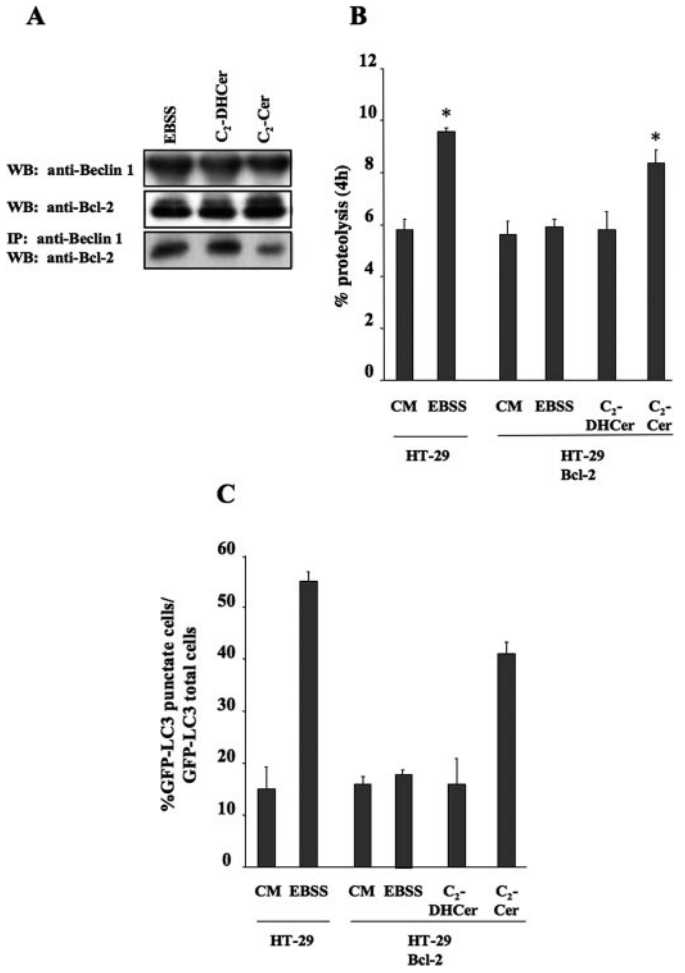

FIGURE 4. **C_2-Cer induces both autophagy and Beclin/Bcl-2 complex dissociation in HT-29 cells.** *A*, HT-29 Bcl-2 cells were cultured for 4 h in EBSS, or complete medium (*CM*) supplemented with 100 μM C_2-Cer or 100 μM C_2-DH Cer. Endogenous Beclin 1 was immunoprecipitated using a goat polyclonal antibody (1:80 dilution). Immunoprecipitated proteins were subjected to immunoblotting using polyclonal anti-Bcl-2 antibody. Lysates were immunoblotted using polyclonal anti-Bcl-2 or polyclonal anti-Beclin 1 antibodies. *B*, proteolysis (see "Material and Methods") was measured in HT-29 cells treated for 4 h with CM or EBSS; or in HT-29 Bcl-2 cells treated with CM, EBSS, 100 μM C_2-DHCer, or 100 μM C_2-Cer. *C*, HT-29 and HT-29 Bcl-2 cells were transfected with a plasmid encoding GFP-LC3. 24 h after transfection, HT-29 cells were cultured for 4 h either in EBSS or CM. HT-29 Bcl-2 cells were treated for 4 h with CM, EBSS, 100 μM C_2-DHCer, or 100 μM C_2-Cer. Autophagy was quantified by counting the number of cells with GFP-LC3 puncta. The result shown is representative of three independent experiments. 50–100 cells were analyzed per assay. Values reported are the mean ± S.D. of three independent experiments. *Asterisks* indicate $p < 0.05$ *versus* CM.

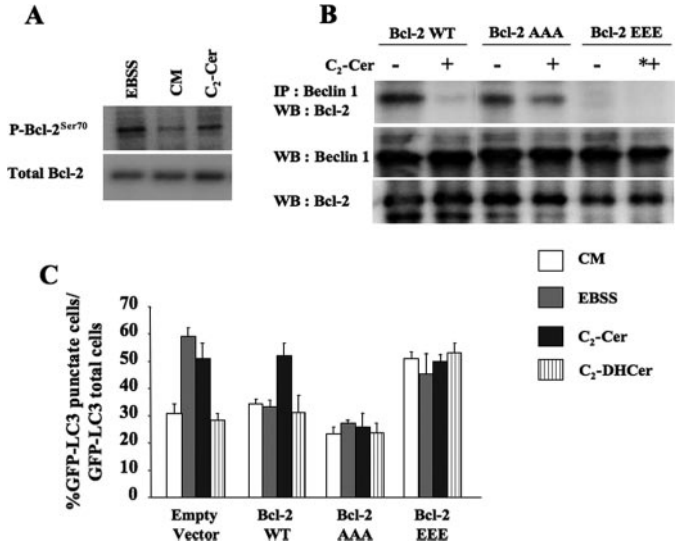

FIGURE 5. **C_2-Cer stimulates Bcl-2 phosphorylation in MCF-7.*beclin 1* cells.** *A*, MCF-7.*beclin 1* cells were cultured for 4 h in EBSS, in complete medium (*CM*) or in CM supplemented with 100 μM C_2-Cer. Lysates were subjected to immunoblotting using a monoclonal antibody against phospho-Bcl-2(Ser^{70}) (1:1000, *upper panel*), or a mouse monoclonal anti-Bcl-2 antibody (1:1000, *lower panel*). *B*, MCF-7.*beclin 1* cells were transfected with a plasmid encoding for Bcl-2 WT, Bcl-2 AAA, or Bcl-2 EEE. 24 h after transfection, the cells were placed for 4 h in CM or 100 μM C_2-Cer. Beclin 1 was immunoprecipitated using goat polyclonal antibody (1:80 dilution). The immunoprecipitated proteins were subjected to immunoblotting using polyclonal anti-Bcl-2 antibody. Lysates was immunoblotted using a polyclonal anti-Bcl-2 or a polyclonal anti-Beclin 1 antibodies. *C*, MCF7.*beclin 1* cells were cotransfected with a plasmid encoding GFP-LC3, an empty vector, or a plasmid encoding for Bcl-2 WT, Bcl-2 AAA, or Bcl-2 EEE. 24 h after transfection, the cells were cultured for 4 h in EBSS, CM, 100 μM C_2-Cer, or 100 μM C_2-DHCer. Autophagy was quantified by counting the number of cells with GFP-LC3 puncta. The result shown is representative of four independent experiments ± S.D. 50–100 cells were analyzed per assay.

Ceramide-induced Autophagy Is Dependent on Bcl-2 Phosphorylation—We then investigated the mechanism that regulates the ceramide-dependent dissociation of the Beclin 1-Bcl-2 complex. Phosphorylation is one of the post-translational changes known to regulate protein complexes. For example, in the autophagic pathway, the recruitment of partners of Atg1 is phosphorylation-dependent (3). Moreover, ceramide modulates the phosphorylation of Bcl-2 (56), and it has been shown recently that Bcl-2 phosphorylation is essential both for the stimulation of starvation-induced autophagy, and for the regulation of the Beclin 1:Bcl-2 rheostat (40). In a first series of experiments, we analyzed the phosphorylation status of Bcl-2 in MCF-7.*beclin 1* cells treated with C_2-Cer using a commercial antibody that detects phosphorylation of the Ser residue at position 70. We observed an increase in the phosphorylation at Ser^{70} after exposure to 100 μM C_2-Cer for 4 h (Fig. 5*A*). We also found that starvation induced greater increase of Ser^{70} phosphorylation than CM (see Ref. (40) and Fig. 5*A*). Ser^{70} is located within a non-structural loop of Bcl-2 characterized by the inclusion of a triad of amino acids Thr^{69}, Ser^{70}, and Ser^{87}, known to be substrates for kinases (57). These same three residues play a major role in Beclin 1-Bcl-2 dissociation in response to starvation (40). To investigate whether these phosphorylation sites are involved in regulating ceramide-induced autophagy and in the dissociation of the Beclin 1-Bcl-2 complex, we transfected MCF-7.*beclin 1* cells with the cDNAs that encode wild-type Myc-Bcl-2, Myc-Bcl-2AAA (where Thr^{69}, Ser^{70}, and Ser^{87} were mutated to Ala by site-directed mutagenesis), and Myc-Bcl-2EEE (where Thr^{69}, Ser^{70}, and Ser^{87} were mutated to Glu by site-directed mutagenesis).

As illustrated in Fig. 5 (*B* and *C*), in contrast to starvation (40), C_2-Cer stimulated autophagy and induced the dissociation of the Beclin 1-Bcl-2 complex in MCF7.*beclin 1* cells expressing the wild-type Myc-Bcl-2. These results are consistent with those obtained in HT-29-Bcl-2 cells (see Fig. 4, *A–D*). As observed in HT-29-Bcl-2 cells, C_2-DHCer did not induce autophagy in MCF-7.*beclin 1* cells. C_2-Cer treatment did not induce dissociation of the Beclin 1-Bcl-2 complex in MCF-7.*beclin 1* cells transfected with the Myc-Bcl-2AAA mutant (Fig. 5*B*

and Ref. 40). It was therefore not surprising to find that the autophagic response to C_2-Cer treatment and to starvation was blunted in these cells (Fig. 5*C*). In contrast, in MCF-7.*beclin 1* cells expressing the Myc-Bcl-2EEE mutant, which mimics the phosphorylated form of Bcl-2, dissociation of the Beclin 1-Bcl-2 complex was observed even when autophagy was not stimulated by either C_2-Cer treatment or starvation (Fig. 5*B* and Ref. 40). The autophagy rate under these conditions was similar to that observed after C_2-Cer treatment or starvation (Fig. 5*C*). This indicates that autophagy cannot be further stimulated by starvation or C_2-Cer once the Beclin 1-Bcl-2 complex has been dissociated. It has been shown recently that simultaneous mutation of the Thr^{69}, Ser^{70}, and Ser^{87} of Bcl-2 to Ala resulted in a constitutive interaction between Beclin 1 and Bcl-2 during periods of starvation (see Ref. 40). In contrast, simultaneous mutation of the Thr^{69}, Ser^{70}, and Ser^{87} of Bcl-2 to Glu alters the interaction between Beclin 1 and Bcl-2. Accordingly, the effects of ceramide treatment on Beclin 1-Bcl-2 dissociation and the autophagic response shown in Fig. 5 were only observed when all three residues in the non-structural loop of Bcl-2 were mutated to Glu or to Ala (data not shown).

JNK1 Regulates Ceramide-induced Autophagy and Ceramide-dependent Bcl-2 Phosphorylation—Residues in the non-structural loop are substrates for various different Ser/Thr protein kinases (57). Among the kinases that phosphorylate the three residues in the non-structural loop, JNK1 has been shown to phosphorylate all three residues (57, 58). Moreover, preliminary results showed that inhibitors of PKC and p38 MAPK are not able to mitigate the Beclin 1-Bcl-2 dissociation induced by C_2-Cer (data not shown). We therefore first analyzed the phosphorylation of JNK1 in HeLa GFP-LC3 cells. C_2-Cer treatment triggered the activation of JNK1 after incubating for 4 h (Fig. 6*A*), by which time the level of long-chain ceramides was significantly increased (see Fig. 1*D*). It is important to note that incubating for 2 h did not modify either Bcl-2 phosphorylation or JNK1 phosphorylation (data not shown), which strengthens the idea that long-chain ceramide synthesis is essential. In addition, we confirmed that starvation stimulated both Bcl-2 phosphorylation (see Fig. 5*A*) and JNK1 phosphorylation (Fig. 6*A*). Following these results, we expressed a dominant-negative form of JNK1. When this mutant was expressed in HeLa GFP-LC3 cells, ceramide-induced autophagy and starvation-induced autophagy were both totally blocked (Fig. 6*B*), suggesting that JNK1 activation is an essential step in the stimulation of autophagy. Moreover, in this context, the increase in Bcl-2 phosphorylation was blocked both in C_2-Cer-treated cells and starved cells (Fig. 6*C*).

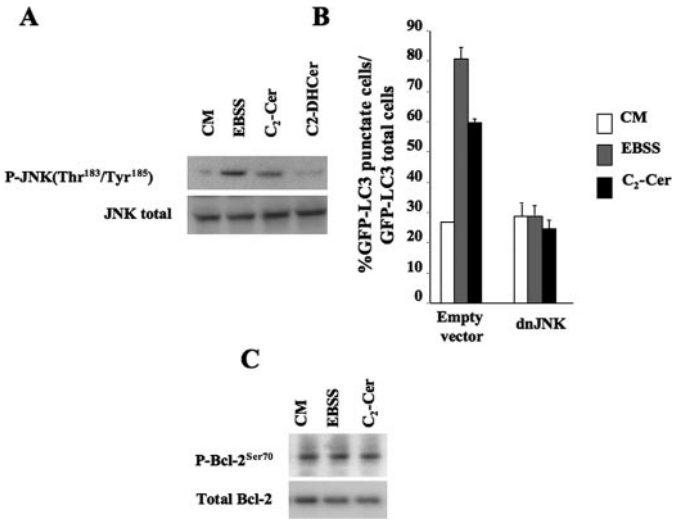

FIGURE 6. **C_2-Cer stimulates autophagy via JNK1 activity in HeLa cells.** *A*, HeLa cells were cultured for 4 h in EBSS, complete medium (*CM*) alone or supplemented with 100 μM C_2-Cer. Lysates were immunoblotted with a rabbit monoclonal anti-Phospho-JNK(Thr^{183}/Tyr^{185}) antibody (1:1000, *upper panel*) or a rabbit monoclonal anti-JNK antibody (1:1000, *lower panel*). *B*, HeLa cells were co-transfected with a plasmid encoding for GFP-LC3, an empty vector or a vector encoding inactive JNK1 (*dnJNK1*). 24 h after being transfected, the cells were treated for 4 h with EBSS, CM, or 100 μM C_2-Cer. Autophagy was quantified by counting the number of cells with GFP-LC3 puncta. The result shown is representative of three independent experiments. 50–100 cells were analyzed per assay. *C*, HeLa cells were transfected with a plasmid encoding dnJNK1. 4 h after being transfected, the cells were treated for 4 h with EBSS, CM, 100 μM C_2-Cer, or 100 μM 100 μM C_2-DHCer. The cells were then subjected to electrophoresis and immunoblotting using rabbit monoclonal anti-Phospho-JNK(Thr^{183}/Tyr^{185}) antibody (1:1000), a rabbit monoclonal anti-JNK antibody (1:1000), or a monoclonal antibody raised against phospho-Bcl-2(Ser^{70}) (1:1000, *upper panel*) or a mouse monoclonal anti-Bcl-2 antibody (1:1000). Values reported are the mean ± S.D. of three independent experiments.

DISCUSSION

Ceramide is a sphingolipid second messenger involved in a wide variety of cell processes (28, 31). A better understanding of its role in cellular homeostasis calls for characterization of its intracellular targets. In the work reported here, we identified the Beclin 1-Bcl-2 complex involved in the early stages of autophagosome formation of as a new target for ceramide. In this complex, the anti-apoptotic protein Bcl-2 has an anti-autophagic function (19), and so dissociation of the complex is required to trigger autophagy during starvation to promote cell survival in the absence of nutrients (19). In addition to physiological stimuli, such as starvation or the production of ceramide, drugs relevant to cancer therapy, such as the BH3-mimetic ABT-747, have been shown to dissociate the Beclin 1-Bcl-2 complex by competing with the BH3 domain of Beclin 1 (25).

Based on the present and previous studies (36), we conclude that ceramide stimulates autophagy by interfering with the activities of the mTORC1 complex and of the Beclin1 complex. This situation is reminiscent of that observed during starvation-induced autophagy, where the activity of both these complexes is modulated (23). Interestingly, the Beclin 1 protein is known to act as a scaffold protein that directly or indirectly recruits several partners, including oncoproteins (Bcl-2 and Bcl-X_L) and tumor suppressors (UVRAG, Ambra-1, and Bif-1) (22, 23). Oncoproteins have an inhibitory effect on the activity of hVps34 (class III phosphatidylinositol 3-phosphate) in the complex, and consequently on autophagy. Tumor suppressors have the opposite effects on both autophagy and hVps34 activity. In this context, it is interesting to note that ceramide, which is a tumor suppressing lipid (31), induces the dissociation of the Beclin 1-Bcl-2 complex and stimulates autophagy.

Ceramide-induced dissociation of the Beclin 1-Bcl-2 complex requires the phosphorylation of Bcl-2. Previous studies have shown that ceramide modulates the anti-apoptotic capacity of Bcl-2 by decreasing its phosphorylation as a result of acti-

vating protein phosphatase 2A (56). This would suggest that ceramide triggers autophagy by inhibiting the anti-autophagic function of Bcl-2 via its phosphorylation and apoptosis by dephosphorylating Bcl-2. As far as the anti-autophagic function of Bcl-2 is concerned, the phosphorylation of the non-structural loop alleviates its anti-autophagic capacity, as shown in response to starvation or to ceramide (Ref. 40 and the present study). Deletion of the non-structural loop of Bcl-2 blocks both starvation-induced autophagy (see Ref. 40) and ceramide-induced autophagy (data not shown). As discussed elsewhere (59), the absence of the structural loop in viral forms of Bcl-2 is probably a strategy for viruses to block the induction of autophagy during cell infection. Interestingly, deleting this non-structural loop and the absence of phosphorylation of amino acids in this loop both reinforce the anti-apoptotic function of cellular Bcl-2 (58, 60).

The subcellular localization of Bcl-2 in ceramide-induced autophagy and ceramide-induced apoptosis is probably an important parameter. Mitochondria have been shown to play a major role in ceramide-induced apoptosis (61). Furthermore, a mitochondrial protein phosphatase 2A has been implicated in the ceramide-dependent dephosphorylation of Bcl-2 that triggers apoptosis (56). These findings suggest that the mitochondrial-targeted Bcl-2 plays a critical role in regulating ceramide-induced apoptosis. In contrast, only the ER-targeted form of Bcl-2 regulates autophagy (19, 25). Phosphorylation of ER-located Bcl-2 inhibits its binding to pro-apoptotic family members (62) and induces its dissociation from Beclin 1 (40).

The great plasticity of sphingolipid metabolism means that ceramide can be produced via several different routes (*de novo* synthesis, hydrolysis of sphingomyelin, and degradation of glycolipids) in different subcellular locations (63). It is interesting to observe that an increase in the *de novo* synthesis of ceramide in the ER in response to Tam treatment leads to the dissociation of the Beclin 1-Bcl-2 complex. This could suggest that the ER pool of ceramide could probably be involved in modulating autophagy via the Beclin 1-Bcl-2 complex. The transport of ceramide out of the ER is dependent on the CERT protein (64). Inhibition of CERT expression is known to induce an ER stress response (65). On the other hand, in different settings ER stress is known to induce autophagy (66, 67). It is tempting to envisage the possibility that ceramide could be a part of the signaling that triggers ER-dependent autophagy. However, it is likely that the ceramide produced by the recycling salvage pathway of sphingosine out of the ER (53) may also interfere with the composition of the Beclin 1-Bcl-2 complex. It would be worth investigating the contributions of the various different pools of ceramide in the induction of autophagy, to see whether this would shed any light on the roles played by the different pools of this lipid in regulating autophagy and apoptosis. Interestingly, a recent reports shows that CD95-dependent ceramide can trigger both pro-survival signals (autophagy) and pro-death signals (apoptosis) in carcinoma cells (68), suggesting that ceramide cannot be considered to be solely a mediator of cell death.

Ceramide mediates the dissociation of the Beclin 1-Bcl-2 complex by stimulating the phosphorylation of Bcl-2 by JNK1. JNKs, which are stress-activated protein kinases, can be activated by ceramide (69, 70). One effect of this ceramide-dependent activation of JNKs is the phosphorylation of Bcl-2 family members (71). JNKs are a key regulator of the balance between cell survival and cell death. The duration of activation, as well as the nature of the substrate, are key elements in the equilibrium between the pro- or anti-apoptotic activities of JNK (72). The ceramide-dependent activation of autophagy via JNK is another parameter involved in the pro-survival and pro-death activity of JNK, because of the dual role of autophagy in cell death and survival.

During this work we noticed that ceramide was more potent than starvation in dissociating the Beclin 1-Bcl-2 complex. In fact, ceramide induced dissociation of the complex and stimulated autophagy in a context of the forced expression of Bcl-2 (HT-29-Bcl-2 cells and MCF-7.*beclin 1*-Bcl-2 cells), whereas in this context, starvation had no effect on Beclin 1-Bcl-2 dissociation and did not stimulate autophagy (40). This difference between the effects of starvation and ceramide is probably not the consequence of distinct signaling pathways. In fact, both stimuli inhibit the mTOR signaling pathway, which acts upstream of the Beclin 1-Bcl-2 complex during the formation of autophagosomes (17, 36). One possible explanation for the robust effect of ceramide on the dissociation of the Beclin 1-Bcl-2 complex is that, in addition to Bcl-2 phosphorylation, ceramide may also promote the dissociation of the complex by other mechanisms. Ceramide has been shown to trigger autophagy via the induction of the BH3-only protein BNIP3 (37). BNIP3 began to accumulate in response to ceramide after 4–5 h of treatment, *i.e.* on a time scale compatible with the experiments performed here. In addition, several reports indicate that BNIP3 is involved in the induction of autophagy (37, 73–75). A recent study has shown that, during hypoxia, BNIP3 is able to displace Bcl-2 from the Beclin 1 complex, probably by competing with the BH3-domain of Beclin 1 (75). This means that we cannot exclude the possibility that ceramide may use two strategies to dissociate Bcl-2 from Beclin1: first, by increasing its phosphorylation of Bcl-2 via JNK1, and second by favoring the BNIP3-Bcl-2 complex rather than the Beclin 1-Bcl-2 complex. One question that remains to be answered is how ceramide leads to an accumulation of *Bnip3*. Recently, the expression of several autophagy genes, including BNIP3, has been shown to be under the control of the transcription factor FOXO3 (73). FOXO3 is inhibited by Akt/PKB-dependent phosphorylation (76), but this inhibitory effect can be overcome by ceramide, which inhibits Akt/PKB (77). Moreover, as we have reported elsewhere, ceramide increases the level of Beclin 1 mRNA (36). So we hypothesize that the ceramide-induced inhibition of Akt/PKB may stimulate autophagy by two, non-mutually exclusive pathways: by interfering with the activation of the mTORC1 complex and by regulating the expression of autophagy genes via FOXO3.

The present findings suggest that ceramide is a second messenger with a central role in the regulation of autophagy. Elucidation of the targets of ceramide in the autophagic pathway will clarify its role in cell homeostasis. Together with our previous results showing that another sphingolipid second messenger, sphingosine 1-phosphate, also regulates autophagy (35), it is clear that the plasticity of sphingolipid metabolism means that there are a variety of possible mechanisms for triggering auto-

phagy in response to stress situations and to influence cell fate (78).

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REFERENCES

1. Levine, B., and Klionsky, D. J. (2004) *Dev. Cell* **6,** 463–477
2. Mizushima, N., Levine, B., Cuervo, A. M., and Klionsky, D. J. (2008) *Nature* **451,** 1069–1075
3. Xie, Z., and Klionsky, D. J. (2007) *Nat. Cell Biol.* **9,** 1102–1109
4. Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., and Mizushima, N. (2006) *Nature* **441,** 885–889
5. Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E., and Tanaka, K. (2006) *Nature* **441,** 880–884
6. Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., Ohsumi, Y., Tokuhisa, T., and Mizushima, N. (2004) *Nature* **432,** 1032–1036
7. Lum, J. J., Bauer, D. E., Kong, M., Harris, M. H., Li, C., Lindsten, T., and Thompson, C. B. (2005) *Cell* **120,** 237–248
8. Boya, P., Gonzalez-Polo, R. A., Casares, N., Perfettini, J. L., Dessen, P., Larochette, N., Metivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P., and Kroemer, G. (2005) *Mol. Cell. Biol.* **25,** 1025–1040
9. Levine, B. (2007) *Nature* **446,** 745–747
10. Mathew, R., Karantza-Wadsworth, V., and White, E. (2007) *Nat. Rev. Cancer* **7,** 961–967
11. Maiuri, M. C., Zalckvar, E., Kimchi, A., and Kroemer, G. (2007) *Nat. Rev. Mol. Cell. Biol.* **8,** 741–752
12. Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C. B., and Tsujimoto, Y. (2004) *Nat. Cell Biol.* **6,** 1221–1228
13. Yu, L., Alva, A., Su, H., Dutt, P., Freundt, E., Welsh, S., Baehrecke, E. H., and Lenardo, M. J. (2004) *Science* **304,** 1500–1502
14. Djavaheri-Mergny, M., Amelotti, M., Mathieu, J., Besancon, F., Bauvy, C., Souquere, S., Pierron, G., and Codogno, P. (2006) *J. Biol. Chem.* **281,** 30373–30382
15. Espert, L., Denizot, M., Grimaldi, M., Robert-Hebmann, V., Gay, B., Varbanov, M., Codogno, P., and Biard-Piechaczyk, M. (2006) *J. Clin. Invest.* **116,** 2161–2172
16. Qu, X., Zou, Z., Sun, Q., Luby-Phelps, K., Cheng, P., Hogan, R. N., Gilpin, C., and Levine, B. (2007) *Cell* **128,** 931–946
17. Codogno, P., and Meijer, A. J. (2005) *Cell Death Differ.* **12,** Suppl. 2, 1509–1518
18. Gozuacik, D., and Kimchi, A. (2007) *Curr. Top. Dev. Biol.* **78,** 217–245
19. Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X. H., Mizushima, N., Packer, M., Schneider, M. D., and Levine, B. (2005) *Cell* **122,** 927–939
20. Levine, B., and Yuan, J. (2005) *J. Clin. Invest.* **115,** 2679–2688
21. Ohsumi, Y. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 211–216
22. Cao, Y., and Klionsky, D. J. (2007) *Cell Res.* **17,** 839–849
23. Pattingre, S., Espert, L., Biard-Piechaczyk, M., and Codogno, P. (2008) *Biochimie* (*Paris*) **90,** 313–323
24. Erlich, S., Mizrachy, L., Segev, O., Lindenboim, L., Zmira, O., Adi-Harel, S., Hirsch, J. A., Stein, R., and Pinkas-Kramarski, R. (2007) *Autophagy* **3,** 561–568
25. Maiuri, M. C., Le Toumelin, G., Criollo, A., Rain, J. C., Gautier, F., Juin, P., Tasdemir, E., Pierron, G., Troulinaki, K., Tavernarakis, N., Hickman, J. A., Geneste, O., and Kroemer, G. (2007) *EMBO J.* **26,** 2527–2539
26. Oberstein, A., Jeffrey, P. D., and Shi, Y. (2007) *J. Biol. Chem.* **282,** 13123–13132
27. Levine, B., Sinha, S., and Kroemer, G. (2008) *Autophagy* **4,** 600–606
28. Hannun, Y. A., and Obeid, L. M. (2008) *Nat. Rev. Mol. Cell. Biol.* **9,** 139–150
29. Hla, T. (2004) *Semin. Cell Dev. Biol.* **15,** 513–520
30. Kolesnick, R. (2002) *J. Clin. Invest.* **110,** 3–8
31. Ogretmen, B., and Hannun, Y. A. (2004) *Nat. Rev. Cancer* **4,** 604–616
32. Spiegel, S., and Milstien, S. (2003) *Nat. Rev. Mol. Cell. Biol.* **4,** 397–407
33. Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brügger, B., and Simons, M. (2008) *Science* **319,** 1244–1247
34. Hannun, Y. A. (1996) *Science* **274,** 1855–1859
35. Lavieu, G., Scarlatti, F., Sala, G., Carpentier, S., Levade, T., Ghidoni, R., Botti, J., and Codogno, P. (2006) *J. Biol. Chem.* **281,** 8518–8527
36. Scarlatti, F., Bauvy, C., Ventruti, A., Sala, G., Cluzeaud, F., Vandewalle, A., Ghidoni, R., and Codogno, P. (2004) *J. Biol. Chem.* **279,** 18384–18391
37. Daido, S., Kanzawa, T., Yamamoto, A., Takeuchi, H., Kondo, Y., and Kondo, S. (2004) *Cancer Res.* **64,** 4286–4293
38. Zeng, X., Overmeyer, J. H., and Maltese, W. A. (2006) *J. Cell Sci.* **119,** 259–270
39. Zheng, W., Kollmeyer, J., Symolon, H., Momin, A., Munter, E., Wang, E., Kelly, S., Allegood, J. C., Liu, Y., Peng, Q., Ramaraju, H., Sullards, M. C., Cabot, M., and Merrill, A. H., Jr. (2006) *Biochim. Biophys. Acta* **1758,** 1864–1884
40. Wei, Y., Pattingre, S., Sinha, S., Bassik, M., and Levine, B. (2008) *Mol. Cell* **30,** 678–688
41. Chaumorcel, M., Souquere, S., Pierron, G., Codogno, P., and Esclatine, A. (2008) *Autophagy* **4,** 46–53
42. Furuya, N., Yu, J., Byfield, M., Pattingre, S., and Levine, B. (2005) *Autophagy* **1,** 46–52
43. Bielawska, A., Perry, D. K., and Hannun, Y. A. (2001) *Anal. Biochem.* **298,** 141–150
44. Lei, K., Nimnual, A., Zong, W. X., Kennedy, N. J., Flavell, R. A., Thompson, C. B., Bar-Sagi, D., and Davis, R. J. (2002) *Mol. Cell. Biol.* **22,** 4929–4942
45. Bauvy, C., Meijer, A. J., and Codogno, P. (2008) *Methods Enzymol.*, in press
46. Seglen, P. O., and Gordon, P. B. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 1889–1892
47. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000) *EMBO J.* **19,** 5720–5728
48. Tanida, I., Yamaji, T., Ueno, T., Ishiura, S., Kominami, E., and Hanada, K. (2008) *Autophagy* **4,** 131–134
49. Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y., and Yoshimori, T. (2001) *J. Cell Biol.* **152,** 657–668
50. Liang, X. H., Yu, J., Brown, K., and Levine, B. (2001) *Cancer Res.* **61,** 3443–3449
51. Mizushima, N., and Yoshimori, T. (2007) *Autophagy* **3,** 542–545
52. Merrill, A. H., Jr. (2002) *J. Biol. Chem.* **277,** 25843–25846
53. Kitatani, K., Idkowiak-Baldys, J., and Hannun, Y. A. (2008) *Cell Signal.* **20,** 1010–1018
54. Bleicher, R. J., and Cabot, M. (2002) *Biochim. Biophys. Acta* **1585,** 172–178
55. Ogier-Denis, E., Couvineau, A., Maoret, J. J., Houri, J. J., Bauvy, C., De Stefanis, D., Isidoro, C., Laburthe, M., and Codogno, P. (1995) *J. Biol. Chem.* **270,** 13–16
56. Ruvolo, P. P., Deng, X., Ito, T., Carr, B. K., and May, W. S. (1999) *J. Biol. Chem.* **274,** 20296–20300
57. Blagosklonny, M. V. (2001) *Leukemia* **15,** 869–874
58. Yamamoto, Z., Ichijo, H., and Korsmeyer, S. J. (1999) *Mol. Cell. Biol.* **19,** 8469–8478
59. Liang, C., Xiaofie, E., and Jung, J. U. (2008) *Autophagy* **4,** 268–272
60. Maundrell, K., Antonsson, B., Magnenat, E., Camps, M., Muda, M., Chabert, C., Gillieron, C., Boschert, U., Vial-Knecht, E., Martinou, J.-C., and Arkinstall, S. (1997) *J. Biol. Chem.* **272,** 25238–25242
61. Birbes, H., El Bawab, S., Obeid, L. M., and Hannun, Y. A. (2002) *Adv. Enzyme Regul.* **42,** 113–129
62. Bassik, M. C., Scorrano, L., Oakes, S. A., Pozzan, T., and Korsmeyer, S. J. (2004) *EMBO J.* **23,** 1207–1216
63. Futerman, A. H., and Hannun, Y. A. (2004) *EMBO Rep.* **5,** 777–782
64. Hanada, K., Kumagai, K., Yasuda, S., Miura, Y., Kawano, M., Fukasawa, M., and Nishijima, M. (2003) *Nature* **426,** 803–809
65. Swanton, C., Marani, M., Pardo, O., Warne, P. H., Kelly, G., Sahai, E.,

Elustondo, F., Chang, J., Temple, J., Ahmed, A. A., Brenton, J. D., Downward, J., and Nicke, B. (2007) *Cancer Cell* **11,** 498–512
66. Høyer-Hansen, M., and Jäättelä, M. (2007) *Cell Death Differ.* **14,** 1576–1582
67. Yorimitsu, T., and Klionsky, D. J. (2007) *Trends Cell Biol.* **17,** 279–285
68. Park, M. A., Zhang, G., Martin, A. P., Hamed, H., Mitchell, C., Hylemon, P. B., Graf, M., Rahmani, M., Ryan, K., Liu, X., Spiegel, S., Norris, J., Fisher, P. B., Grant, S., and Dent, P. (2008) *Cancer Biol. Ther.* **7,** 1648–1662
69. Basu, S., and Kolesnick, R. (1998) *Oncogene* **17,** 3277–3285
70. Saslowsky, D. E., Tanaka, N., Reddy, K. P., and Lencer, W. I. (2008) *FASEB J.*, in press
71. Kurinna, S., Tsao, C. C., Nica, A. F., Jiffar, T., and Ruvolo, P. P. (2004) *Cancer Res.* **64,** 7852–7856
72. Liu, J., and Lin, A. (2005) *Cell Res.* **15,** 36–42
73. Mammucari, C., Milan, G., Romanello, V., Masiero, E., Rudolf, R., Del Piccolo, P., Burden, S. J., Di Lisi, R., Sandri, C., Zhao, J., Goldberg, A. L., Schiaffino, S., and Sandri, M. (2007) *Cell Metabol.* **6,** 458–471
74. Tracy, K., Dibling, B. C., Spike, B. T., Knabb, J. R., Schumacker, P., and Macleod, K. F. (2007) *Mol. Cell. Biol.* **27,** 6229–6242
75. Zhang, H., Bosch-Marce, M., Shimoda, L. A., Tan, Y. S., Baek, J. H., Wesley, J. B., Gonzalez, F. J., and Semenza, G. L. (2008) *J. Biol. Chem.* **283,** 10892–10903
76. Burgering, B. M., and Medema, R. H. (2003) *J. Leukoc. Biol.* **73,** 689–701
77. Ruvolo, P. P. (2003) *Pharmacol. Res.* **47,** 383–392
78. Lavieu, G., Scarlatti, F., Sala, G., Levade, T., Ghidoni, R., Botti, J., and Codogno, P. (2007) *Autophagy* **3,** 45–47